Claims 1-3, 32-52, 56, and 57 are rejected under 35 U.S.C. § 112, first paragraph, as being enabling for a method of removing amyloid deposits from the TRIAD mouse, but does not reasonably provide enablement for a method of removing amyloid deposits from a subject.

The present invention is based in part on the finding that immunization with amyloid fibrils can result in the induction of an immune response that promotes removal of amyloid deposits, including deposits comprising fibril subunits other than that used as the immunogen. These amyloid fibrils from various diseases differ considerably from each other in their primary sequence.

In addition to the data presented in the instant application including the TRIAD mouse model data, other data confirm the results presented herein. For instance, it has been shown that the monoclonal antibody (mAb) 11-1F4 expedited the resolution of implanted human immunoglobulin light chain (AL), amyloidomas in mice by inducing targeted, cell-mediated dissolution (Hrncic *et al.*, 2000, Am. J. Path. 157(4): 1239 (Attached to Amendment)). MAb 11-F4 was generated using as an immunogen, heat-denatured κ4 immunoglobulin light chain protein, combined with adjuvant. It was also shown that the therapeutic efficacy of the mAb 11-1F4 was independent of the Vκ/Vλ subgroup of the injected amyloid which suggests that the monoclonal antibody was interacting with a conformational (structural) epitope shared by different amyloid fibrils composed of immunoglobulin light chains (Hrncic *et al.*, 2000, Am. J. Path. 157(4): 1239). In an extension of this hypothesis, the mAb 11-1F4 expedited the removal of systemic AA amyloid deposits, composed of serum amyloid protein A, in a murine model of inflammation-associated amyloidosis (Wall *et al.*, 2001, Amyloid and Amyloidosis: Proceedings of the IXth International Symposium on Amyloidosis, Budapest, Hungary, David Apathy (Attached to Amendment)), which provides evidence that the conformational epitope recognized by mAb 11-1F4 may be common to many types of amyloid fibril, other than those composed of light chain proteins.

Data supporting the existence of a generic, fibril-associated epitope has also been recently presented by O'Nuallain *et al.* who demonstrated reactivity of two IgM mAbs, WO1 and WO2, with synthetic fibrils composed of Aβ, poly-glutamine, and LC fragments. WO1 and WO2 are generated from the fibrillar form of Aβ(1-40). In each case, these mAbs did not bind

the non-polymerized precursor proteins Aβ(1-40) proteins (O'Nuallain *et al.*, Proc. Natl. Acad. Sci. 2002, 99(3): 1485-90 (Attached to Amendment)). Accordingly, these publications provide evidence that a fibril-specific epitope exists; that the fibril-specific epitope is related to the three dimensional structure of the fibril; that it can be used to generate "anti-fibril" antibodies that do not react with the non-polymerized precursor protein; and that the anti-fibril antibodies can bind to fibrils composed of structurally unrelated precursor proteins.

The Office Action states that the specification only discloses cursory conclusions without data supporting the findings that amyloid fibrils promote the removal of *in vivo* amyloid fibrils. Applicants respectfully point out that Example D on page 35 discloses data supporting the removal of amyloid deposits from mice. The mice used in Example D were not TRIAD mice. These mice were immunized with synthetic fibrils, and were shown to have anti-fibril antibodies. On day 59 of the immunization protocol, the mice were administered a subcutaneous bolus of human AL amyloid extract to yield an AL amyloidoma. As discussed in Example D, the amyloidoma disappeared within 5 days from these mice. The data of Example D show the removal of *in vivo* amyloid deposits from a subject comprising administering to the subject an effective amount of the amyloid fibrils. Accordingly, the specification provides data and working example to support the breadth of the claimed invention. Thus, Applicants respectfully request withdrawal of this rejection.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 1, 2, 32-45, 50-52, 56, and 57 have been rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim Applicants' invention.

Claims 1 and 50 have been amended to more distinctly point out the claimed invention. Accordingly, the claims, as they stand, are not indefinite. Applicants respectfully request the withdrawal of the rejection.

Claim 37 has been rejected as being indefinite because the use of the term "cystatin C variant". However, it is well known that the cystatin C variant associated with amyloidosis causes Hereditary Cystatin C Amyloid Angiopathy (HCCAA) and that HCCAA is caused by a

mutation in the gene encoding the peptidase inhibitor cystatin C. Thus, it is known that the term "cystatin C variant" refers to the Leu68Q variant form of cystatin C. (See attached Grubb *et al.*, http://www.reasearch.swegene.org/project_details.php?Proj=194) Accordingly, claim 37 is definite as it stands.

Rejections Under 35 U.S.C. §102

A. Claims 1, 3, 32-40, 46, 56, and 57 are rejected under 35 U.S.C. §102(b) as being anticipated by Kline *et al.* (WO 95/31996).

The Office Action states that Kline *et al.* teach a method for alleviating the symptoms of a disease state associated with plaque formation such as Alzheimer's disease by administering to a patient amyloid beta protein or a derivative thereof. Applicants respectfully point out that the claims of the present application are directed to a method of removing amyloid deposits from a subject comprising administering amyloid fibrils. Unlike the method of treatment disclosed by Kline *et al.*, the present application provides a method of removing amyloid deposits from a subject comprising administering a composition of amyloid fibrils. The amyloid fibrils induce an immune response in a subject which promotes the removal of amyloid deposit. Amyloid fibrils and the amyloid beta protein of Kline *et al.* are structurally and functionally distinct products. Moreover, the specific use of amyloid fibrils is not anticipated by the work of Kline *et al.* As described in paragraphs 78 and 79 on pages 21 and 22, amyloid fibrils are protein aggregates of proteins or peptides that may differ from those in the amyloid deposits of the subject. The amyloid beta protein of Kline *et al.* is a single protein. Thus, claims 1, 3, 32-40, 46, 56, and 57 are not anticipated by Kline *et al.* Applicants respectfully request withdrawal of this rejection.

B. Claims 53 and 54 are rejected under 35 U.S.C. §102(b) as being anticipated by Ostberg *et al.* (US Patent 5,750,106).

Applicants respectfully point out that claims 53 and 54 are directed to a vaccine or pharmaceutical composition comprising amyloid fibrils which contain immunoglobulin light chain polypeptide or a whole immunoglobulin light chain peptide. Applicants submit that

Ostberg *et al.* do not disclose a pharmaceutical composition comprising amyloid fibrils which contain immunoglobulin light chain. The pharmaceutical composition of Ostberg *et al.* comprises a human anti-CMV antibody and a buffer. The pharmaceutical composition of Ostberg *et al.* and the pharmaceutical composition of claims 53 and 54 are different because they comprise structurally and functionally distinct active ingredients. As discussed above, an amyloid fibril is a structured protein aggregate, unlike the human anti-CMV antibody of Ostberg *et al.* Thus, claims 53 and 54 are not anticipated by Ostberg *et al.* Applicants respectfully request withdrawal of this rejection.

C. Claims 1, 3, 32-49, 56, and 57 are rejected under 35 U.S.C. 102(a) as being anticipated by Schenk *et al.* (WO 99/27944).

The Office Action states that Schenk *et al.* teach a method for treating patients suffering from amyloidogenic disease, such as Alzheimer's, comprising administering amyloid-beta peptide or variants thereof to induce an immune response against the amyloid deposits in the patient. Applicants respectfully submits that the claims of the present application are directed to a method of removing amyloid deposits from a subject comprising administering amyloid fibrils to the subject. As discussed above, amyloid fibrils and the amyloid-beta peptide or variants thereof are structurally and functionally distinct products. Thus, the claims are not anticipated by Schenk *et al.* Accordingly, Applicants respectfully request the withdrawal of this rejection.

Rejection Under 35 U.S.C. §103(a)

Claims 53-55 are rejected under 35 U.S.C. §103(a) as being unpatentable over Ostberg *et al.* (US Patent 5,750,106) and Theofan *et al.* (US Patent 5,643,570).

Applicants respectfully points out that claim 53 is directed to a pharmaceutical composition comprising amyloid fibrils that contain immunoglobulin light chain polypeptide or whole immunoglobulin light chain polypeptide. Dependent claims 54 and 55 includes carrier and an adjuvant, respectively. As discussed above, Ostberg *et al.* do not disclose pharmaceutical compositions comprising amyloid fibrils. Likewise, Theofan *et al.* do not teach a pharmaceutical composition comprising amyloid fibrils. The pharmaceutical composition of Ostberg *et al.*

comprises anti-CMV antibody, and the pharmaceutical composition of Theofan *et al.* contains BPI-immunoglobulin and carriers, adjuvants, and diluents. Anti-CMV antibody and BPI-immunoglobulin are structurally and functionally distinct from amyloid fibrils because amyloid fibrils are structured protein aggregates. Thus, the combination of the cited references do not render claims 53-55 obvious. Accordingly, Applicants respectfully request the withdrawal of this rejection.

CONCLUSION

In view of the foregoing claim amendments and accompanying remarks, Applicants respectfully request reconsideration and timely allowance of the pending claims. Should the Examiner feel that there are any issues outstanding after consideration of this response, the Examiner is invited to contact Applicants' undersigned representative to expedite prosecution.

If there are any additional fees due in connection with the filing of this response, please charge the fees to our Deposit Account No. 50-0310. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

Claim 1 has been amended as follows:

1. (Twice Amended) A method of removing amyloid deposits from a subject comprising administering to the subject amyloid fibrils in an effective amount to generate an immune response, wherein the immune response promotes the removal of amyloid deposits from the [~~patient~~] subject.

Claim 50 has been amended as follows:

50. (Amended) A method of removing amyloid deposits from a subject comprising administering to the subject amyloid fibrils comprising an immunoglobulin light chain polypeptide and or a whole immunoglobulin light chain polypeptide in an effective amount to generate an immune response, wherein the immune response promotes the removal of amyloid deposits from the [~~mammal~~] subject.

Claim 53 has been amended as follows:

53. (Amended) A vaccine or pharmaceutical composition of claim 3 comprising an immunoglobulin light chain polypeptide or a whole immunoglobulin light chain polypeptide.

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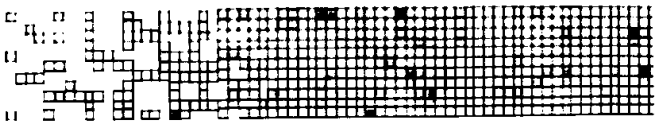

CYSTATINS AS REGULATING ELEMENTS AND PATHOPHYSIOLOGICAL MARKERS

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We sequenced in 1981 a protein with unknown function, now called cystatin C, which defined a new superfamily of cysteine protease inhibitors ("cystatins"). We have described four new cystatins and shown that cystatin C is produced by a house-keeping gene and that a mutation in it causes the dominantly inherited disease "Hereditary Cystatin C Amyloid Angiopathy" in which L68Q-cystatin C is precipitated as amyloid in affected individuals, who die from brain hemorrhage before 40 years of age. We demonstrated in 1984 that the serum level of cystatin C probably is a better marker for glomerular filtration rate than serum creatinine.

We and others have recently shown that cystatin C might regulate antigen presentation, is a growth factor for mouse neural stem cells, and might be involved in the atherosclerotic process. We have lately produced mice with deleted cystatin C genes, which we now will use to investigate in more detail the roles of cystatin C as a modulator of antigen presentation, particularly in experimental rheumatoid arthritis, as a brain growth factor, and as a factor in the pathophysiology of atherosclerosis.

We determined in 2001 the crystal structure of human cystatin C, which may be used to understand the molecular pathophysiology of cystatin C amyloidosis and perhaps for design of drugs against amyloidosis in general. These possibilities will

now be tested using the transgenic mice carrying human L68Q-cystatin C genes that we created in 2002.

We have shown that small peptidyl derivatives, based upon the structure of cystatins, have antiviral and antibacterial effects and will now try to elucidate their antibacterial and antiviral mechanisms in an effort to create new classes of antibacterial and antiviral drugs.

We will also try to identify those clinical situations in which serum cystatin C will be a particularly important marker for specific renal dysfunction in an effort to substitute non-invasive for invasive diagnostic procedures for kidney disease.

Publications

Janowski R, Kozak M, Jankowska E, Grzonka Z, Grubb A, Abrahamson M, Jaskolski M
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Cystatin C deficiency in human atherosclerosis and aortic aneurysms
J Clin Invest 1999; 104:1191-1197

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Total financing:
3.9 MSEK

Gov grant for clinical research ("ALF"):
2.6 MSEK

Total external financing:
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Natl and intl prioritized grants:
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Project homepage:

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Antibody-Mediated Resolution of Light Chain-Associated Amyloid Deposits

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Primary light-chain-associated (AL) amyloidosis is characterized by the deposition in tissue of monoclonal light chains as fibrils. With rare exception, this process is seemingly irreversible and results in progressive organ dysfunction and eventually death. To determine whether immune factors can effect amyloid removal, we developed an experimental model in which mice were injected with amyloid proteins extracted from the spleens or livers of patients with AL amyloidosis. Notably, the resultant amyloidomas were rapidly resolved, as compared to controls, when animals received injections of an anti-light-chain monoclonal antibody having specificity for an amyloid-related epitope. The reactivity of this monoclonal antibody was not dependent on the V_L or C_L isotype of the fibril, but rather seemed to be directed toward a β-pleated sheet conformational epitope expressed by AL and other amyloid proteins. The amyloidolytic response was associated with a pronounced infiltration of the amyloidoma with neutrophils and putatively involved opsonization of fibrils by the antibody, leading to cellular activation and release of proteolytic factors. The demonstration that AL amyloid resolution can be induced by passive administration of an amyloid-reactive antibody has potential clinical benefit in the treatment of patients with primary amyloidosis and other acquired or inherited amyloid-associated disorders. *(Am J Pathol 2000, 157:1239–1246)*

Primary amyloidosis is a monoclonal plasma cell dyscrasia characterized by the pathological deposition as fibrils of immunoglobulin light-chain-related components (ie, AL amyloid) in the heart, kidney, liver, tongue, nerves, and other anatomical sites throughout the body.[1–4] The relentless accumulation of fibrillar protein within these tissues leads to progressive organ dysfunction and eventually death.[5] Heretofore, treatment of patients with this devastating disorder has focused on reducing the synthesis of amyloidogenic precursor light chains using antiplasma cell chemotherapy given in conventional or, more recently, in high doses combined with autologous stem cell transplantation.[5–15] Such efforts have extended survival and, in some cases, resulted in improvement of organ function over time.[13–15] However, certain patients, eg, the elderly or those with extensive cardiac amyloid deposition, are not candidates for such intensive therapy and their prognoses remain exceedingly poor.[11–15] More recently, the administration of an experimental chemotherapeutic agent, the doxorubicin analog 4-DOX, was found serendipitously to accelerate removal of AL amyloid deposits without seemingly decreasing the bone-marrow plasma-cell population or the concentration of the precursor monoclonal Ig.[17] Although this compound binds to various types of amyloid,[18] the process that leads to resorption of fibrils is unknown. Further, the clinical usefulness of 4-DOX is limited because of its hematological toxicity and the fact that the most striking therapeutic responses have occurred in patients with soft-tissue amyloid deposits, whereas little or no improvement has been noted in those with heart, kidney, or liver involvement.[19]

Amyloid deposition, thus, is not necessarily an irreversible process.[20–23] In the case of AL, the existence of endogenous mechanisms that can effect amyloid removal has been evidenced by the finding that proteins extracted from pathological deposits most often consist of fragments formed from the degradation of the carboxy-terminal portion of their precursor light chain molecules, presumably by neutrophil-derived proteases.[1] That AL fibrils are not eliminated totally may result from their nonself-origin nature and the body's consequent failure to mount an effective immune response to this material. Additionally, the presence of other noncollagenous co-deposited with amyloid (eg, P component[24] and certain glycosaminoglycans[24–32]) has been alleged to interfere with amyloidolysis.[26–33]

To investigate factors that could promote amyloid resorption, we have developed an *in vivo* experimental model involving mice in which amyloidomas were produced by

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the subcutaneous injection of human AL extracts. We now report the results of studies in which it was shown that this material was in fact removed by an immune mechanism associated with the formation of anti-amyloid antibodies and a resultant neutrophil cellular reaction. Based on these observations, we have generated a murine monoclonal antibody (mAb) that recognizes an epitope present on AL amyloid fibrils, as evidenced by enzyme-linked immunosorbent assay (ELISA), immunoblotting, and immunohistochemistry. This reagent, when administered to mice bearing human AL amyloidomas, bound to the fibrils and elicited a neutrophil response. Notably, this process resulted in rapid and complete elimination of the amyloid tumors as compared to untreated animals. The demonstration that this anti-amyloid antibody can effect amyloidolysis *in vivo* provides a potentially novel means of therapy for patients with primary amyloidosis.

Materials and Methods

Amyloid Extraction and Chemical Characterization

The method used to prepare water-soluble amyloid extracts was essentially that described by Pras et al.[illegible] Briefly, 30 to 40 g of fresh-frozen (−80°C) or 10 g of lyophilized spleen or liver obtained postmortem from patients with AL amyloidosis were homogenized in ~400 ml of cold saline with a Virtis-Tempest apparatus (Virtis, Gardner, NY). The homogenates were centrifuged at 6°C for 30 minutes at 17,000 rpm and residual saline-soluble material was removed by repeated homogenization and washing until the resultant supernatant had an OD of <0.1 at A_{280}. The pellet was then repeatedly homogenized, washed with cold deionized water, centrifuged, and the amyloid-containing supernatants lyophilized. The amount of protein recovered represented approximately one-third to one-fifth the weight of the starting material.

The light chain composition and V_L subgroup of the amyloid was determined by amino acid sequencing (Procise Protein Sequencing System; Applied Biosystems, Foster City, CA) and ionizing mass spectroscopy (PE SCIEX API 150 EX; Perkin Elmer, Norwalk, CT) of high-performance liquid chromatography-separated peptides obtained by trypsin digestion of reduced and pyridylethylated protein[illegible] extracted from the water-soluble material with 6 mol/L guanidine HCl. The presence of the proteoglycan heparan sulfate was established using an Azure A assay.[illegible]

Monoclonal Antibody

A lyophilized sample of a V_κ fragment derived from enzymatic (pepsin) cleavage of a κ4 Bence Jones protein[illegible] was dissolved in a 1 mol/L sodium acetate buffer, pH 4.8, to a final concentration of 1 mg/ml, precipitated from solution by heat treatment at 56°C for 15 minutes, and resuspended in phosphate-buffered saline (PBS) before injection into BALB/c mice. The techniques used to generate and characterize the murine mAb 11-1F4 were as previously described,[33,34] as were those to fluorescein-label, obtain through pepsin digestion an F(ab')₂ fragment, and biotinylate the antibody.[35]

Immunochemical Assays

For solid-phase ELISA, 96-well flat-bottomed microwell plates (Corning CoStar, Corning, NY) were filled with 50 μl of a 10 μg/ml solution of human light chain containing fibrils extracted from amyloidotic livers and spleens or with recombinant V_L fibrils[illegible] and allowed to dry overnight by incubation at 37°C. After blocking and washing, appropriately diluted samples of mouse serum, culture fluid supernatant, or purified mAb were added to each well. Detection of bound antibody was accomplished using a peroxidase-labeled goat anti-mouse IgG antiserum (BioRad, Richmond, CA) and a 2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid] substrate solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Color development was terminated after 15 minutes by the addition of 2% oxalic acid and measured at an OD of 415 nm using an ELISA plate reader (Bio-Tek Instruments, Winooski, VT).

For Western blotting, amyloid extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, using the NuPAGE electrophoresis system (Novex, San Diego, CA). The proteins were electrotransferred to 0.45-μm nitrocellulose membranes (Millipore, Bedford, MA) and, after blocking, were exposed to mouse sera, purified murine anti-light chain mAbs,[31] or an anti-P component reagent (Calbiochem, La Jolla, CA). The blots were washed and treated with an alkaline phosphatase-labeled horse anti-mouse IgG antiserum (ABC Kit; Vector Laboratories, Burlingame, CA). Bound protein was detected with the Western Blue Stabilized Substrate (Promega, Madison, WI). Immunohistochemical analyses were performed using the ABC technique as specified by the manufacturer (Vector) on 4-μm-thick deparaffinized tissue sections mounted on poly-L-lysine-coated slides. The primary and secondary antibodies and substrate used were 11-1F4, an affinity-purified goat anti-mouse IgG horseradish-peroxidase conjugate (Bio-Rad), and diaminobenzidine (Vector), respectively.

Microscopy

Four- to 6-μm-thick tissue sections were cut for light microscopy. Stains for leukocyte esterases were performed using naphthol AS-D chloroacetate esterase and α-naphthyl acetate solutions (Sigma Diagnostics, St. Louis, MO) according to the manufacturer's directions. To detect amyloid, the sections were treated with a freshly prepared alkaline Congo red solution and viewed under polarized light using a Wild microscope (Leitz, Rockleigh, NJ) with a gypsum plate and a filter analyzer. For electron microscopy, precipitates were applied to formvar carbon-coated copper grids, air dried, stained with 1% phosphotungstic acid, and viewed with a Hitachi H-600

electron microscope (Hitachi Science Systems, Ltd., Ibaraki, Japan).

Amyloidoma Formation

Lyophilized water-soluble amyloid extracts were suspended in 0.25 ml of sterile saline and homogenized with a PCU-2 Polytron apparatus (Brinkman Lucerne, Switzerland). The fibrils were sedimented by centrifugation at 4°C for 30 minutes at 17,000 rpm; the resultant pellet was resuspended in 1 ml of sterile saline and rehomogenized. This solution was injected subcutaneously between the scapulae of mice using an 18-gauge needle attached to a 1-cm³ syringe. The size of the resultant amyloidoma was measured by daily palpation and confirmed at necropsy. High-resolution X-ray-computed tomography images were acquired using a microCat apparatus (Oak Ridge National Laboratory, Oak Ridge, TN).

Mice

BALB/c, IFO-18 null, and C.B-17 SCID mice were purchased from Charles River Laboratories (Wilmington, MA), Jackson Laboratories (Bar Harbor, ME), and Taconic (Germantown, NY), respectively. All mice were treated in accordance with National Institutes of Health regulations under the aegis of a protocol approved by the University of Tennessee's Animal Care and Use Committee.

Results

To investigate humoral or cellular factors that can facilitate amyloid removal, we developed an *in vivo* experimental model in which 6-week-old BALB/c mice were injected subcutaneously between the scapulae with 50 to 200 mg of water-soluble AL amyloid extracts. The composition of this material was established by chemical, immunoblotting, amino acid sequence, and ionizing mass spectroscopic analyses where the predominant protein species were found to be κ or λ light chain-related molecules that, in most cases, consisted primarily of the variable region (V_L) plus the first ~50 residues of the constant region (C_L) and, in others, V_L fragments or intact molecules. Additionally, these extracts contained the expected amyloid-associated P-component,[23] as well as the proteoglycan heparan sulfate.[24] The injected material formed a readily visible, palpable mass on the backs of animals, the size of which depended on the amount of material injected (eg, 0.2 to 2.5 cm in maximum diameter). The amyloidoma remained localized and unchanged for ~10 to 24 days, as evidenced by high-resolution X-ray-computed tomography; after that point, the tumors began to regress and eventually disappeared throughout an ~4-day period (Figure 1). This response occurred regardless of the κ or λ nature or the V_L subgroup of the amyloid extract; however, in studies involving five different κ and seven λ amyloidomas, ALλ extracts typically resolved more slowly than did ALκ (ALλ, 18 ± 6 days *versus* ALκ, 13 ± 3 days). Sufficient material was available to repeat experiments at least four times in eight of the 12 cases where it was found that this effect was reproducible in healthy, young animals regardless of the tissue source of the amyloid. However, dissolution of the induced amyloid-

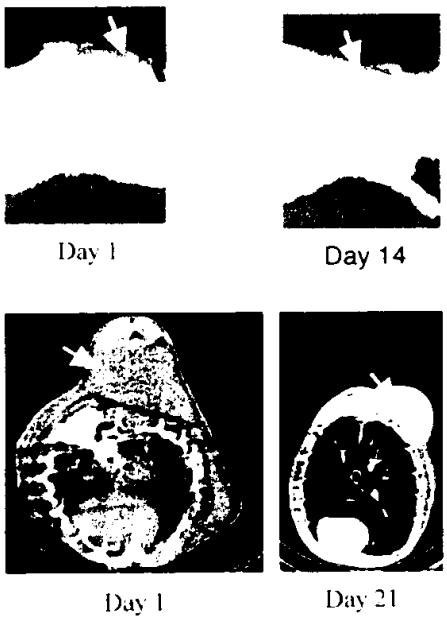

Figure 1. Human AL amyloidoma model. **Top:** Appearance of a mouse injected subcutaneously between the scapulae with 200 mg of a human ALκ amyloid extract (**left**) and resolution of the amyloidoma after 14 days (**right**). **Bottom:** Radiographic CT scan images of a mouse after injection of a human ALλ amyloid extract (**left**) and 21 days later (**right**).

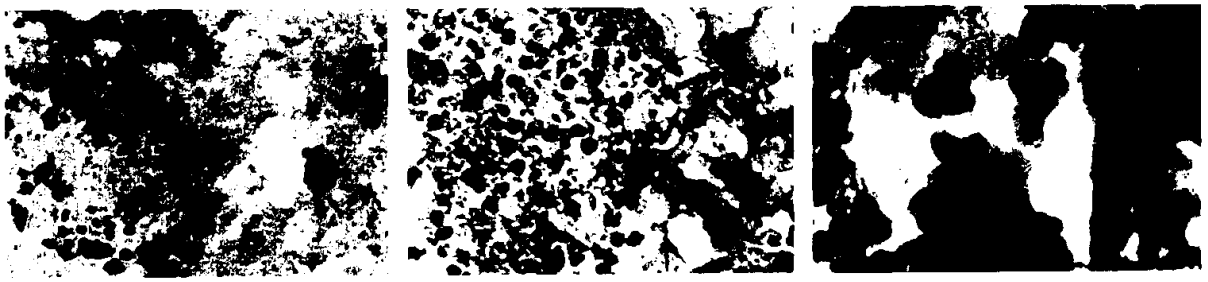

Figure 2. Infiltration of regressing amyloidoma by polymorphonuclear leukocytes. Amyloid tumor excised on day 10 and formalin-fixed, paraffin-embedded sections stained with Congo red (**left**), hematoxylin-eosin (**middle**), and naphthol AS-D chloroacetate (**right**). Original magnifications: ×400, ×400, ×1000, respectively.

omas was consistently delayed beyond 3 months in aged (> 18 months) and immunodeficient (SCID) mice.

Histological studies to determine the fate of the regressing amyloidomas demonstrated that the amyloid was not redistributed to other mouse tissues, as evidenced by Congo red staining. Additionally, the tumors were infiltrated by naphthol AS-D chloroacetate-positive, α-naphthyl acetate-negative, polymorphonuclear cells, ie, neutrophils (Figure 2). In contrast, this cellular response did not occur in CD-18 null mice[30] where resolution of human AL amyloidomas required a considerably longer time period (ie, >3 months). Further, amyloidolysis was delayed in animals rendered profoundly neutropenic by the administration of 250 μg of the anti-neutrophil mAb RB6-8C5[29] given at the time of amyloidoma induction and again on day 5.

Amyloid removal also was dependent on a humoral immune response to the human light-chain containing material. Approximately 10 to 20 days after amyloidoma induction, we showed in immunoblotting experiments that mouse sera contained antibodies that recognized not only the light chain constituent of the amyloid protein injected, but also that of heterologous ALκ or ALλ extracts (Figure 3). In contrast, there was no reactivity with the homologous amyloid precursor protein, ie, Bence Jones protein or any other monoclonal light chain tested. When the same amyloid preparation was re-administered to these immunized animals, its rate of disappearance increased approximately twofold. Additionally, in other experiments, elimination of amyloid tumors was accelerated when the extracts were incubated overnight with mouse immune serum before injection. A similar response occurred when serum-treated extracts were injected into SCID mice.

Based on these experiments, we hypothesized that the seemingly irreversible nature of amyloid deposition results from the patient's inability to elicit an immune response directed toward this material. We thus reasoned that, if available, the passive administration of anti-amyloid antibodies could expedite amyloid resolution and, thus, potentially provide a new therapeutic approach to this disease. To test this theory, we injected mice with a V$_L$ fragment obtained by proteolytic cleavage of a human κ Bence Jones protein[31] that, on thermal denaturation under acidic conditions, formed material that possessed the characteristic features of amyloid,[32] namely, after Congo red staining it exhibited green birefringence when viewed by polarizing microscopy and, by electron microscopy, appeared fibrillar. Spleen cells harvested from the immunized animals were fused with SP2 cells and culture fluid supernatants from the resultant hybridomas were screened for reactivity in a solid-phase ELISA[33] using glass-capture proteins κ and λ amyloid fibrils. Selected hybridoma colonies were injected intraperitoneally into pristane-primed BALB/c mice and the resultant mAbs purified from ascitic fluid by gel filtration. Among those tested, one, an IgG1 antibody (designated 11-1F4), exhibited the greatest degree of reactivity with both types of molecules. Notably, the binding of mAb 11-1F4 was unrelated to the V$_L$ or C$_L$ isotypic properties of the amyloid proteins tested, ie, there was no correlation between

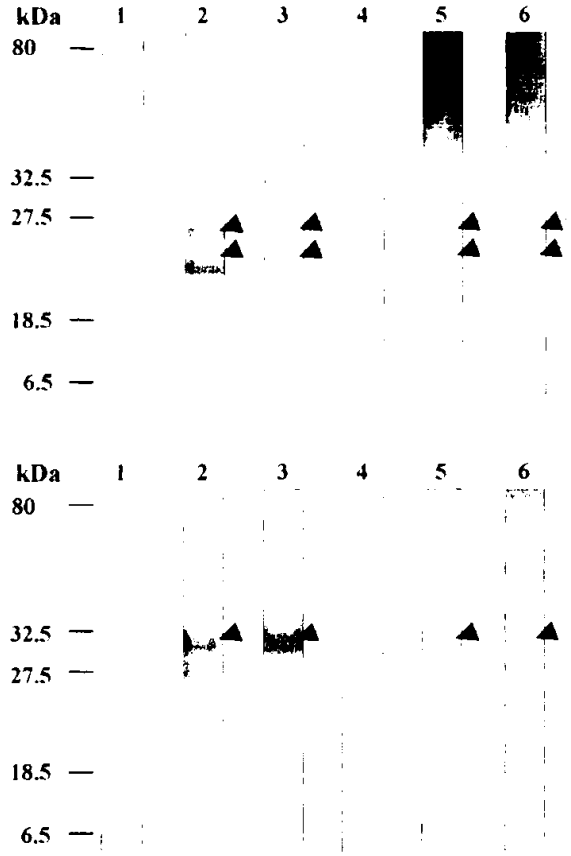

Figure 3. Humoral immune response to AL amyloidoma. Detection by immunoblotting of murine anti-human AL fibril antibodies. **Top:** ALκ HIG amyloid extract stained with Coomassie blue (**lane 2**) and blotted with an anti-κ light chain mAb (**lane 3**), non immune serum obtained from a normal mouse (**lane 4**), serum obtained from a mouse 20 days after HIG amyloidoma induction (**lane 5**), and serum obtained from a mouse 20 days after BAL amyloidoma induction (**lane 6**). **Bottom:** ALλ BAL extract stained with Coomassie blue (**lane 2**) and blotted with an anti-λ light chain mAb (**lane 3**), nonimmune serum obtained from a normal mouse (**lane 4**), serum obtained from a mouse 20 days after BAL amyloidoma induction (**lane 5**), and serum obtained from a mouse 20 days after HIG amyloidoma induction (**lane 6**). The M_rs of the molecular mass markers located in **lane 1** are indicated in kd.

the V$_κ$/V$_λ$ subgroup or C$_κ$/C$_λ$ nature of the light chain constituent and the interaction with this antibody. Alternatively, when tested in a liquid-phase ELISA[33] against a panel of Bence Jones proteins representative of the four major V$_κ$ and five V$_λ$ subgroups, this reagent was specific only for κ4 light chains.

The anti-amyloid reactivity of 11-1F4 also was evidenced immunohistochemically. As shown in Figure 4, both ALκ and ALλ deposits were recognized by this antibody. Most importantly, we found that mAb 11-1F4 bound to AL amyloid *in vivo*. In one such experiment, a mouse injected with an ALκ1 amyloid extract was inoculated concomitantly in the thigh with 100 μg of fluorescein-labeled antibody. The animal was sacrificed 36 hours later and the excised amyloidoma examined by fluorescence microscopy (Figure 5). The labeled antibody localized only to the amyloidoma and not to any mouse tissue. Similarly, this reagent did not react with normal human tissue.

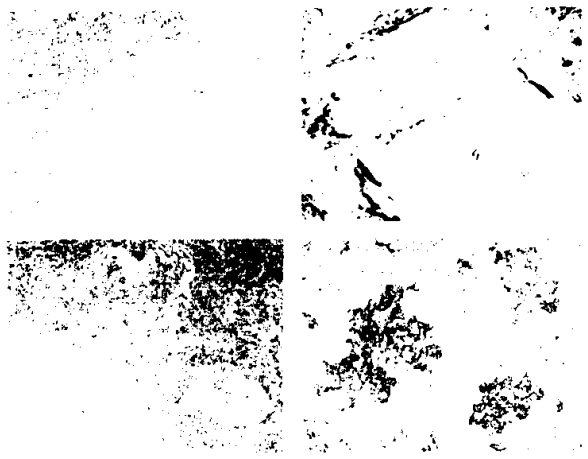

Figure 4. Reactivity of mAb 11-1F4 with ALκ and ALλ amyloid tissue deposits. Histochemical and immunohistochemical analyses of liver and spleen tissue obtained from ALκ1 patient HIG (**top**) and ALλ patient SHE (**bottom**), respectively. **Left:** Polarizing microscopy of Congo red-stained sections. **Right:** Immunoperoxidase staining with the mAb 11-1F4. Original magnifications, ×200.

To test the therapeutic efficacy of mAb 11-1F4, a series of experiments were initiated in which 100-μg doses of reagent were given to pairs of mice bearing human AL amyloidomas. In the case of ALκ studies involving two different extracts (HIG and GRA) revealed that even a single injection of the antibody resulted in rapid and complete disappearance of the amyloid tumor, as compared to untreated animals (Table 1). As illustrated in Figure 6 (top), the mass of an ALκ1 amyloidoma was reduced >90% within 4 days after antibody injection, as compared to control animals. However, to achieve a similar response in certain ALλ-type amyloidomas, multiple doses of the reagent were required. These were given as a series of 100-μg injections beginning at the time when the amyloidoma was induced (day 0) and then again on days 2, 4, and 6 (Figure 6, bottom). As summarized in Table 1, in experiments in which five different human ALλ amyloidomas were tested in the mouse model (JON, SHE, FIE, BUE, and BAL), it was found that treatment with mAb 11-1F4 decreased by as much as fourfold the time in which the amyloid tumors were eliminated. Notably, although single or repeated doses of two other anti-light chain mAbs[25] that recognized AL fibrils (eg, 31-8C7) expedited amyloidolysis, the 11-1F4 reagent was unique in that it accelerated removal of both ALκ and ALλ amyloid. In contrast, three anti-light chain mAbs that lacked such reactivity were ineffective.

In other studies, it was demonstrated that for amyloidolysis to occur, the complete 11-1F4 IgG molecule was required, as evidenced when a biotinylated pepsin-derived F(ab')$_2$ fragment of this antibody, although localizing to the human amyloidoma, did not accelerate amyloidolysis. Further, histological examination of residual amyloid tumors removed from 11-1F4-treated mice revealed the presence of a pronounced neutrophilic infiltration, whereas little or no cellular response was seen in the unresolved material taken from control animals within the same time period (ie, 5 to 6 days). Additionally, based on ELISA and immunoblotting analyses, neither group of mice had detectable serum antibodies to the injected amyloid in these short-term experiments (data not shown).

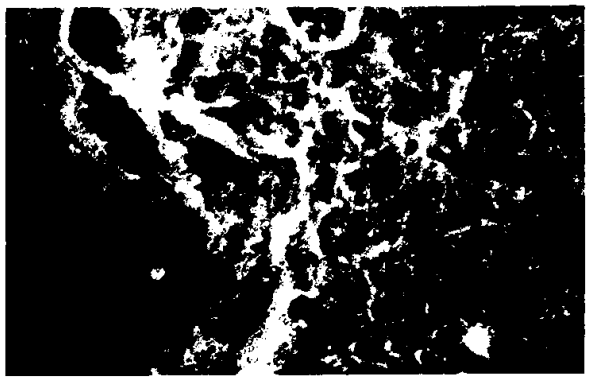

Figure 5. Localization of fluorescein-labeled mAb 11-1F4 within an ALκ amyloidoma. (Fluorescent microscopy; original magnification, ×400).

Discussion

We have shown that amyloidomas formed by subcutaneous injection of human AL amyloid extracts into healthy mice were resolved within 14 to 26 days by an immune-mediated mechanism involving the generation of anti-amyloid antibodies and a resultant polymorphonuclear leukocyte response. The sera obtained from such animals contained antibodies that recognized antigenic determinants not only present on the injected protein, but also common to both ALκ and ALλ amyloid fibrils. Preincubation of the amyloid extracts with immune serum

Table 1. Monoclonal Antibody-Mediated Amyloidolysis in Mice Bearing Human ALκ and ALλ Amyloidomas

Amyloidoma (V$_L$ subgroup)	Single dose* Treated 11-1F4†	Single dose* Treated 31-8C7‡	Single dose* Untreated	Multiple doses* Treated 11-1f4	Multiple doses* Treated 31-8C7	Multiple doses* Untreated
ALκ(1) HIG	4§	12	14	NT¶	NT	NT
ALκ(1) GRA	4	8	15	NT	NT	NT
ALλ(6) JON	8	11	14	NT	NT	NT
ALλ(1) SHE	9	19	21	9	20	24
ALλ(1) FIE	17	24	24	9	18	26
ALλ(2) BUE	24	NT	25	6	NT	25
ALλ(3) BAL	28	NT	28	7	NT	28

*100 μg on day 0; †100 μg on days 0, 2, 4, and 6; ‡mAb designation; §time (days); ¶NT, not tested.

Figure 6. Monoclonal anti-amyloid antibody-mediated resolution of human AL amyloidomas. **Top:** Appearance of residual ALκ amyloid tumor on day 4 in a mouse given a single 100-μg injection of mAb 11-1F4 at the time of amyloidoma induction (**left**) and in an untreated animal (**right**). **Bottom:** Appearance of residual ALλ amyloid tumor on day 7 in a mouse given 100-μg injections of mAb 11-1F4 at the time of amyloidoma induction (day 0) and then again on days 2, 4, and 6 (**left**) and in an untreated animal (**right**).

resulted in more rapid resolution of the induced amyloidomas in normal and SCID mice.

These observations led us to immunize mice with human V_L fibrils and to generate an antibody that recognized an amyloid-related epitope, as demonstrated immunochemically. Based on the pattern of reactivity, we established that this determinant was not necessarily related to the κ or λ nature of the AL fibrils or to the V_L subgroup of the immunogen used to prepare the antibody. Thus, we posited that this anti-amyloid reagent recognized a β-pleated structure common to AL fibrils that such material contains antigenic sites not exposed

on the soluble light chain precursor protein has been shown by other investigators.[40,41] We also found that mAb 11-1F4 recognized other forms of amyloid, as evidenced in immunohistochemical analyses of AA-, ATTR-, ALyS-, AApoA1- and Aβ-containing tissues. In each case, similar patterns of reactivity were obtained with 11-1F4 and antibodies specific for these five different types of amyloid proteins (Wall J, Macy S, Weiss DT, Solomon A, unpublished studies).

The therapeutic potential of mAb 11-1F4 was demonstrated in our *in vivo* experimental model where its administration into normal or SCID mice in which human AL amyloidomas had been induced resulted in marked acceleration of amyloidolysis as compared to untreated animals. This antibody was shown to localize within the amyloid tumors and, further, rapid resolution of this material occurred before the mice could mount a detectable humoral immune response to the human proteins contained within the AL extracts. Although a single 100-μg injection of antibody 11-1F4 resulted in rapid amyloidolysis in mice bearing the two ALκ amyloidomas, for three of the ALλ amyloid tumors studied, several doses were required to achieve complete resolution. The apparent resistance of ALλ versus ALκ fibrillar deposits to immune-mediated lysis also was noted in mice antibody-treated mice. Although it has been postulated that the presence of the amyloid-associated P component or glycosaminoglycans may protect fibrils from degradation by cellular or humoral processes,[26,42] amyloidolysis occurred in both treated and control animals despite the presence of these components in the amyloid extracts used for injection.

Antibody-mediated amyloid resolution in our experimental AL mouse model was associated with an infiltration of neutrophils within the amyloid. The essentiality of these cells in effecting AL amyloid dissolution was demonstrated in studies involving neutropenic as well as CD-18 knockout mice in which a component of the neutrophil β-integrin cell-surface adhesion molecules is lacking and thus, extravascular transpedesis of neutrophils is prevented.[43]

Based on our experimental data, we posit that amyloidolysis results from a three step process that includes: 1) the binding or opsonization of fibrils by the anti-amyloid mAb; 2) attraction and activation of neutrophils via an interaction between their Fcγ receptors and the Fcγ portion of the antibody molecule; and 3) enzymatic and/or chemical proteolysis[35–38] of the amyloid by neutrophil-derived endopeptidases or free radicals, respectively.

Although subcutaneous amyloidomas occur in patients with AL amyloidosis, these often pathological fibrillar deposits are found in organs throughout the body.[7] Because of the lack of a suitable animal model of this disease process, it remains to be determined if administration of an anti-amyloid antibody would expedite lysis of systemically deposited material. However, because mAb 11-1F4 also recognizes other types of amyloid proteins, eg, AA, we have tested it in our transgenic AA amyloidotic mice[45] that develop extensive hepatic fibrillar deposits and found that there was a rapid and marked diminution of this material in the livers of animals given this reagent (Wall J, Schell M, Wooliver C, Wolfenbarger DA, Weiss DT, Solomon A, unpublished studies). Indeed, that humoral immunity may effect amyloidolysis has been inferred from the report by Schenk et al[46] who demonstrated using a transgenic mouse model of Alzheimer's disease that older animals immunized with a synthetic Aβ peptide had considerable reduction in cerebral Aβ amyloid plaques.

The use of anti-amyloid antibodies to effect removal of pathological fibrillar deposits would provide a novel means to treat patients with primary (AL) amyloidosis. Currently efforts are underway to prepare a chimerized or humanized version of the murine 11-1F4 mAb that eventually can be tested clinically. Although conventional or high-dose anti-plasma cell chemotherapy still would be required to eliminate the synthesis of the AL precursor light chain, the previous administration of such a reagent would serve to reduce the total body amyloid burden and possibly improve organ function. The development of therapeutic strategies designed to eliminate pathological fibrillar deposits by passive or active[46] immunotherapy would represent a major advance for patients with primary amyloidosis as well as those with other acquired or inherited amyloid-associated disorders.

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Conformational Abs recognizing a generic amyloid fibril epitope

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Disease-related amyloid fibrils appear to share a common, but poorly understood, structure. We describe here the generation and preliminary characterization of two conformation-specific mAbs, WO1 and WO2, that bind to the amyloid fibril state of the Alzheimer's peptide Aβ(1–40) but not to its soluble, monomeric state. Surprisingly, these Abs also bind to other disease-related amyloid fibrils and amyloid-like aggregates derived from other proteins of unrelated sequence, such as transthyretin, islet amyloid polypeptide, β_2-microglobulin, and polyglutamine. At the same time, WO1 and WO2 do not bind to the native protein precursors of these amyloids, nor do they bind to other kinds of protein aggregates. This new class of Abs associated with a fundamental amyloid-folding motif appear to recognize a common conformational epitope with little apparent dependence on amino acid side chain information. These Abs should contribute to the understanding of amyloid structure, assembly, and toxicity and also may benefit the development of diagnostic and therapeutic agents for amyloid diseases.

Amyloid fibrils are highly insoluble, ordered protein aggregates involved in a number of human diseases (1, 2), including Alzheimer's disease (3) and type II diabetes (4). Although the protein components of amyloid fibrils from various disease states differ considerably from each other in primary sequence, all amyloid fibrils share common features, including a high degree of β-sheet in a classical "cross-β" pattern, a fibrillar morphology in electron microscopy, and the ability to bind and alter the spectroscopic properties of heteroaromatic dyes Congo red and thioflavin T (ThT) (5, 6). Although these common properties suggest that amyloid fibrils must share deeper similarities at the molecular level, the extent of similarity between the polypeptide-folding patterns of different amyloids is unknown. Details of the nature of the amyloid fold remain obscure because of technical limitations to obtaining high resolution structural information on large, insoluble, heterodisperse aggregates.

Although mAbs have previously proved useful in the structural analysis of globular proteins, their use in the characterization of amyloid fibril structure has been limited. Most of the anti-fibril Abs generated in an immune response to fibrils tend to be directed at unstructured portions of the amyloidogenic peptide not involved in fibril structure. In a recent characterization of the Ab response in mice injected with amyloid β protein (Aβ) fibrils, it was found that the majority of the Abs are directed at the N-terminal 12 residues of the peptide and are capable of crossreacting strongly with the monomeric peptide (7). This agrees well with the results of limited proteolysis studies of Aβ fibrils indicating an exposed, unstructured N-terminal region in the aggregate (8). Thus, such Abs tell us about those parts of the amyloidogenic peptide that are not involved in fibril structure but little about the nature of fibril structure itself.

Identification of conformational epitopes in fibrils would therefore add an important new dimension to the structural information on fibrils available through Ab studies. Early experiments with rabbit polyclonal sera suggested that amyloid fibrils possess a nonnative structure and that Abs can be generated that are specific for the amyloidogenic conformation (9). More recently, conformational Abs have been reported that are specific for transthyretin (TTR) amyloid fibrils (10) or for the infectious form of mammalian prions (11).

In addition to the value of anti-fibril Abs as structural probes, the nature of the immune response to amyloid is of special interest because of recent reports of successful active and passive vaccine approaches to slowing and/or reversing amyloid plaque growth and/or its pathological consequences in mouse models of light chain amyloidosis (13, †), Alzheimer's disease (14, 15), and mammalian prion disease (16). Some Abs recognizing Aβ fibrils also appear capable of stimulating fibril disassembly (17) and/or preventing fibril assembly (18) *in vitro*.

We report here the results of hydridoma experiments specifically focused on generating conformation-specific Abs against Aβ fibrils (19). The results suggest the existence of a major conformational epitope present in many amyloid fibrils composed of diverse protein sequences.

Materials and Methods

Materials and General Methods. Aβ(1–40) peptides, as well as the polyglutamine (polyGln) molecule NH_2-KKQ_{42}KK-COOH, were custom synthesized at the Keck Biotechnology Center, Yale University. Chemically synthesized, full-length human islet amyloid polypeptide (IAPP) was a gift from Per Westermark. Recombinant JTO5, an amyloidogenic Ig V_L domain, was a gift of Jonathan Wall. Human β_2-microglobulin (β_2m), human TTR, and chicken lysozyme were purchased from Sigma, as were bovine collagen and acid-soluble calf collagen. The κ light chain IgM mAb was purchased from Calbiochem (catalog no. 401925), and an IgG mAb recognizing the 1–17 sequence of Aβ, MAB1560, was purchased from Chemicon. Gelatin was from Bio-Rad. Trifluoroacetic acid was from Pierce and 1,1,1,3,3,3-hexafluoro-2-isopropyl alcohol (HFIP) from Sigma.

Unless otherwise indicated, all quantitative experimental results shown are from measurements done in triplicate. Error bars in figures represent SDs.

Preparation of Solubilized Peptides and Amyloid Fibrils. Each peptide and protein required customized protocols for fibril formation. With the exception of polyGln aggregates, all amyloid fibrils, as well as collagen and elastin, were sonicated on ice with a probe sonicator for five consecutive 30-sec pulses before immobilization onto plastic microtiter plates.

Aβ peptides were solubilized and aggregated by a variation of the previously described protocol (8, 20). Amyloid fibrils were grown from Aβ(1–40) by incubating a 0.25 mg/ml disaggregated solution of the peptide in PBS containing 0.05% sodium azide (PBSA) at 37°C together with a seed consisting of 0.1% by weight

Abbreviations: PBSA, PBS containing 0.05% sodium azide; polyGln, polyglutamine; IAPP, islet amyloid polypeptide; Aβ, amyloid β protein; TTR, transthyretin; β_2m, β_2 microglobulin; ThT, thioflavin T.

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of sonicated Aβ fibrils. The sample was incubated 5–7 days until fibril growth was judged complete by a ThT assay (21).

The polyGln peptide was dissolved and disaggregated as described previously (22) by using a 1:1 mixture of trifluoroacetic acid and 1,1,1,3,3,3-hexafluoro-2-isopropyl alcohol, and then aggregated by incubation of a 0.05 mg/ml solution in PBSA at 37°C for 2 wk until reaction was judged complete by the ThT assay (21). PolyGln aggregates prepared in this manner exhibit strong β-sheet spectra and a typical amyloid ThT response but exhibit ribbon morphology rather than a classical amyloid morphology; based on these and other criteria, we refer to the aggregates as "amyloid-like" (S. Chen, V. Berthelier, J. B. Hamilton, B.O.N., and R.W., unpublished data).

Human IAPP was solubilized and disaggregated by using 1:1 trifluoroacetic acid/1,1,1,3,3,3-hexafluoro-2-isopropyl alcohol (22). After removal of volatile solvents, the peptide was dissolved in 2 mM NaOH and centrifuged at 20,800 × *g* for 25 min. The supernatant was diluted 1:2 by using a 2 × PBS stock containing 0.1% sodium azide, pH 7.4, to a final concentration of ≈0.25 mg/ml. This solution was used immediately both to make amyloid fibrils and to fix monomers to microtiter plates. Fibrils were grown according to the protocol described above for Aβ.

Fibrils were grown from β_2m and TTR in high salt and low pH as described (23, 24). Lysozyme fibrils were grown by a brief exposure to 65°C followed by incubation at 37°C in high salt at low pH (25).

All of the fibrils made as described above exhibited good amyloid fibril morphology by electron microscopy, with the exceptions that the IAPP aggregates appeared to be a mixture of classical amyloid fibrils and other organized structures (data not shown), and the polyGln aggregates appear to be protofilaments assembled into ribbons (26). All amyloid and amyloid-like aggregates exhibited typical ThT fluorescence (21, 27).

Preparation of Aggregated Carboxymethylated Proteins. Reduction and alkylation of the disulfides of ovalbumin and human serum albumin (HSA) was accomplished by dissolving the native protein to a concentration of 0.5 mg/ml in an argon-purged buffer consisting of 0.1 M Tris·HCl, 6 M guanidine hydrochloride, 2 mM DTT, and 1 mM EDTA, pH 8.5. Reactions were incubated at 37°C for 1.5 h with occasional gentle mixing. Iodoacetic acid was then added to a final concentration of 15 mM, and the reaction was incubated in the dark at room temperature for 1 hr then dialyzed overnight at 4°C against PBSA. Complete cleavage of disulfide bonds was confirmed by mobility shifts in nonreducing SDS/PAGE (28). Complete modification of thiol groups was demonstrated by using 5,5′-dithiobis(2-nitrobenzoic acid) (29). The PBSA solution of reduced and alkylated proteins was subjected to several rounds of freezing and thawing, after which the amount of material collected in a pellet after centrifugation was 50% or more of the entire protein sample. These suspensions (without centrifugation) were used to immobilize modified proteins onto microtiter plates.

Hybridoma Isolation and mAb Purification. Aβ(1–40) fibrils were sonicated as described previously (20). Five standard, female BALB/c mice were immunized with 50 μg/mouse/injection with sonicated fibrils. Two additional injections were given at 2-wk intervals. Bleeds were taken 1 wk after each injection and screened by using a modification of the microplate assay described below. After the third injection, two mice were killed and their spleens used to generate hybridoma cells. Initial screening of clones was performed by testing the ability of membranes containing uniformly deposited monomeric or fibrillar Aβ to bind Abs from an array of clonal supernatants; the bound Abs were detected with secondary Abs against murine Ig. The hybridoma experiments, including the membrane blot survey of initial hybridoma colonies, were conducted according to standard methods (30). Animal work, hybridoma creation, cloning, and preliminary screening were performed by the Hybridoma Development Facility at St. Louis University Health Center, St. Louis.

Clonal supernatants giving good binding to immobilized fibrils even in the presence of 80-fold weight excess monomeric Aβ were considered to be good candidates for conformation-specific Abs and were carried forward in the cloning process. mAbs were produced from stable hybridoma cell lines by growing the cells in high density culture by using CELLline incubator flasks (INTEGRA Biosciences). mAbs were purified from the accumulated Ab-containing supernatants by using a HiTrap IgM purification affinity column (Amersham Pharmacia) followed by a Sephacryl S-300 (Amersham Pharmacia) size exclusion chromatography column (PBSA 4°C). Nonreducing SDS/PAGE analysis confirmed the IgM isotype of the Abs and showed that they were at least 90% pure.

Microtiterplate Assays of Fibril Binding. Mouse sera and hybridoma supernatants were assayed for anti-fibril Abs as follows. First, 100 ng of sonicated Aβ(1–40) fibrils in 100 μl of PBSA was added to each well of a high-binding microtiter plate (Costar) and allowed to dry by incubating uncovered overnight in a 37°C oven. Plates were washed three times with PBSA containing 0.05% Tween 20 (the standard wash procedure for all subsequent steps of the protocol). Wells were blocked with 1% gelatin in PBSA at 37°C for 1 hr. Plates were then incubated with sera or hybridoma supernatants, with or without an 80-fold weight excess of monomeric Aβ(1–40) with respect to immobilized fibrils, for 1 hr at 37°C and then washed three times. The signal was developed by incubation with a biotinylated Ab, followed by treatment with a streptavidin conjugate.

For measuring the IgG response in mouse sera (Fig. 1*A*), the secondary Ab was a mixture of isotype-specific goat anti-mouse IgG Abs (Sigma ISO-2 kit) diluted into 1% gelatin, 0.05% Tween 20, and PBSA. The tertiary Ab was a biotinylated rabbit anti-goat Ig Ab (Vector Laboratories). After incubation with a streptavidin-horseradish peroxidase conjugate (Vector Laboratories), the signal was developed with 3,3′,5,5′-tetramethylbenzidine (TMB, Pierce no. 34021).

For measuring the IgG + IgM response in mouse sera (Fig. 1*B*), the secondary Ab was a biotinylated goat anti-mouse Ig Ab (Sigma), and the signal was developed with streptavidin-horseradish peroxidase as described above. For measuring the IgG + IgM response of hybridoma culture supernatants (Fig. 4), the same secondary Ab was used, but the signal was developed by using a europium-streptavidin conjugate (EG & G Wallac) and counted by using time-resolved fluorescence (31) on a Wallac Victor (2) fluorescence microtiter plate reader.

Characterization of Binding Properties of Purified mAbs. For experiments featuring immobilized ligands, microtiter plate wells were coated either with amyloid fibrils or other aggregates (as described above), or with soluble precursor proteins. For the latter, proteins in 2 mM sodium phosphate buffer, pH 7.4, containing 0.05% sodium azide, were incubated uncovered overnight in a 37°C oven. The low salt minimizes aggregation during the coating process (data not shown). After washing, plates were blocked with 3% BSA in PBSA for 1 h at 37°C. For the assay, Ab solutions were incubated 2 h at 37°C in the wells with 3% BSA in PBSA containing 0.05% Tween 20. Binding was quantified by using a biotinylated secondary Ab as described above. In the case of Aβ fibrils, the ability of an 80-fold weight excess of monomeric peptide to inhibit Ab binding to fibrils was also assessed.

Binding of aggregates and soluble proteins to plastic by the above protocol was essentially quantitative. This was determined as follows. After overnight incubation as described, 50–150 μl of

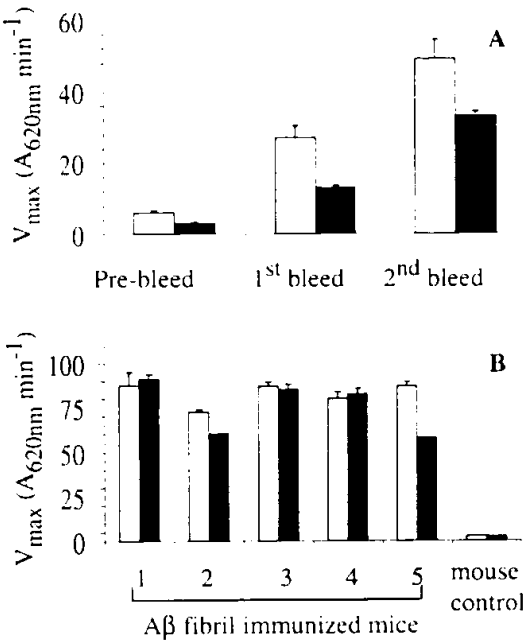

Fig. 1. Serum responses of mice immunized with Aβ(1–40) fibrils. Open bars show total Ig binding to 100 ng/well of amyloid fibrils immobilized onto microtiter plate wells. Closed bars show Ab binding to fibrils in the presence of a large excess of Aβ(1–40) monomer. (*A*) Increase in anti-amyloid IgG response over the course of the immunization treatment. (*B*) Total anti-amyloid IgG + IgM response of five immunized mice and a control at the completion of the immunization protocol.

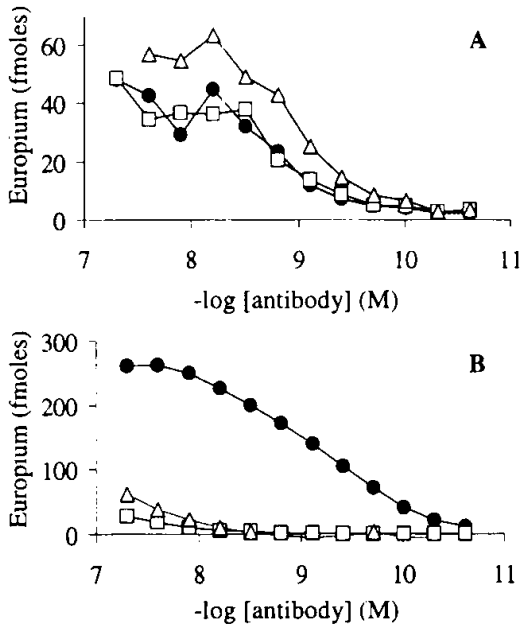

Fig. 2. Binding of purified mAbs to Aβ fibrils in the presence and absence of excess monomeric Aβ. Aβ(1–40) fibrils were immobilized on microplate wells and the wells incubated with anti-Aβ Abs in the absence (●) or presence of monomeric Aβ wild-type (□) or F19P mutant (△). (*A*) Binding of WO1. (*B*) Binding of the IgG mAb MAB1560 against a linear epitope (positions 1–17) of the Aβ(1–40) molecule.

PBS was added to several wells and incubated 10 min at room temperature. Supernatants from several wells were pooled and assayed for the amount of recovered protein, either by a micro protein assay (Pierce MicroBCA) or by recovery of aggregates, solubilization, and quantitation by HPLC against a standard curve (8). Less than 5% of the applied protein was recovered by such analyses, consistent with greater than 95% fixation of aggregates to the plastic.

For experiments featuring immobilized Abs, 100 μl of 15 nM mAb solutions in PBSA were sealed and incubated for 1.5 h at 37°C. Plates were washed and blocked with a 3% BSA solution in PBSA by incubation at 37°C for 1 hr. Various concentrations of biotinyl-Aβ(1–40) were added to each well, and the plate was incubated for 4 h at 37°C and then quantified as before by using streptavidin-europium and time-resolved fluorescence. The N-terminally biotinylated Aβ molecule was prepared by alkylating a Cys^{1} analog of Aβ(1–40) with PEO-iodoacetyl biotin (Pierce).

Results and Discussion

Conformation-specific Abs against the native states of many proteins have been described. Such Abs bind well to the native, folded state of the protein, and less well, or not at all, to the denatured protein or to isolated peptide fragments (32). To isolate Abs specific for conformational epitopes of the Aβ fibril, mice were injected with sonicated Aβ fibrils. These mice mounted a time-dependent serum response consisting of Abs capable of binding to the amyloid form of Aβ immobilized on microtiter plate wells (Fig. 1). Significantly, a major portion of the anti-fibril Ab population binds to Aβ fibrils despite the presence of a large excess of monomeric Aβ (Fig. 1). This suggests that a major portion of the Abs are directed against conformational epitopes that only exist in the fibril. All five mice injected with amyloid exhibit similar serum responses (Fig. 1*B*). Hybridoma fusions were generated from the spleens of responsive mice and resulting cells screened and cloned by using a variety of assays (*Materials and Methods*) to isolate stable cell lines producing Abs that bind to Aβ fibrils but not to Aβ monomers.

Based on results from screening hybridoma supernants, two stable cell lines, designated WO1 and WO2, were selected for further study. Reducing and nonreducing SDS/PAGE (not shown) of cell supernatants showed that both WO1 and WO2 have molecular weights in the 900-kDa range, consistent with the isotyping results on these supernatants showing that both Abs are of the IgM class. Light chains were isolated from the gels and subjected to amino acid sequence analysis. The light chain of WO1 exhibited the N-terminal sequence DIQMTQS, consistent with its being in the κ class of mouse light chains. WO2 appears to consist of a different light chain sequence because it exhibits a blocked N terminus, most consistent with a pyro-Glu residue derived from cyclization of a Gln side chain at position 1.

Fig. 2 compares the fibril-binding characteristics of WO1 to an anti-Aβ IgG mAb that recognizes a primary sequence epitope resident in the 1–17 sequence of Aβ. Fig. 2*A* shows that WO1 exhibits a saturable binding curve against immobilized Aβ fibrils with an EC_{50}, or concentration of half-maximal binding, in the low nanomole range. Fig. 2*A* also shows that when a large weight excess of monomeric Aβ(1–40) is included in the incubation, strong binding to fibrils by WO1 is retained. (The apparent enhancement of binding of WO1 to amyloid fibrils in the presence of wild-type Aβ is probably due to higher fibril mass resulting from the extension of Aβ fibrils by the monomeric Aβ competitor during the incubation.) A soluble, proline-containing mutant incapable of making amyloid fibrils (33, 34), F19P-Aβ(1–40), also does not compete for WO1 binding to fibrils. In

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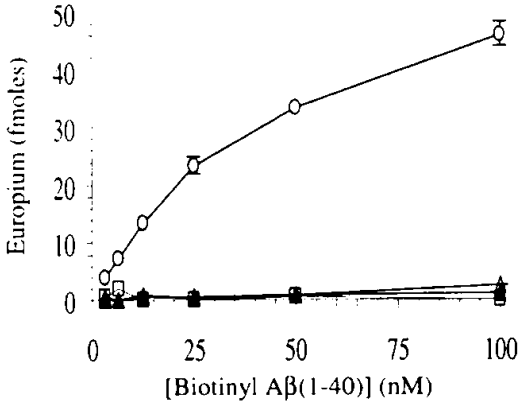

Fig. 3. Binding of monomeric, soluble biotinyl-Aβ to purified Abs immobilized on microplate wells. WO1 ( ); WO2 (▲); control IgM ( ); and anti-Aβ(1–17) IgG MAB1560 ( ).

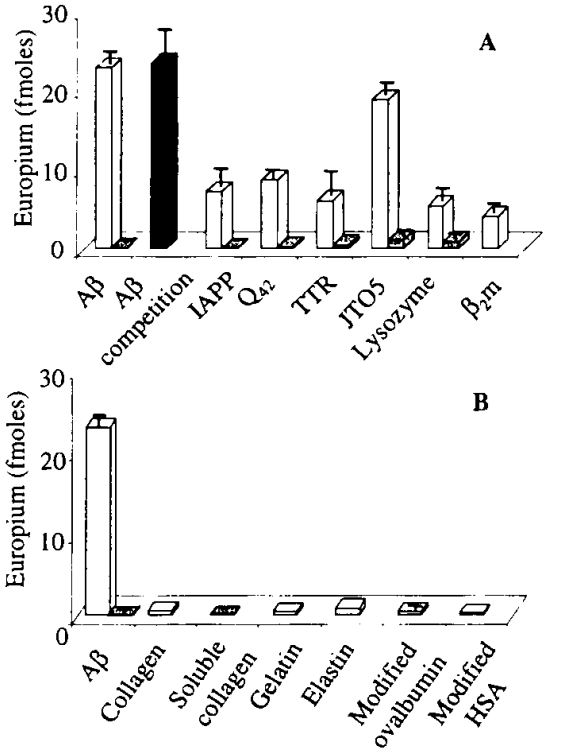

Fig. 4. Binding of WO1 to various aggregates. Open bars, binding to immobilized aggregate; gray bars, binding to immobilized monomeric form; and filled bar, binding to immobilized aggregate in the presence of an 80-fold weight excess monomeric F19P-Aβ(1–40). (*A*) Binding to various amyloids and their precursor proteins. (*B*) Binding to nonamyloid biological aggregates and nonnative globular protein aggregates. In these experiments, WO1 was used as a hybridoma supernatant diluted 1:2 in PBSA.

contrast, both mutant and wild-type monomeric Aβ molecules effectively compete for binding to fibrils by an Ab directed against a linear epitope of Aβ. Fig. 2*B* shows that an Ab directed at the linear 1–17 sequence of Aβ, MAB1560, binds well to immobilized Aβ fibrils. Fig. 2*B* shows that, in contrast to WO1 binding, MAB1560 binding to fibrils can be almost entirely eliminated when a large weight excess of monomeric, soluble Aβ is included in the binding incubation. In these experiments, WO1 and MAB1560 exhibit significantly different binding amplitudes to the same weight of Aβ fibrils; however, given the fact that different secondary Abs were used in these two panels, it is difficult to interpret this effect.

The experiments summarized in Fig. 2 provide indirect evidence that WO1 does not recognize monomeric Aβ. More direct evidence is the demonstration that neither WO1 (Fig. 4) or WO2 (not shown) bind appreciably to monomeric Aβ(1–40) immobilized onto microtiter plate wells. In addition, we find that monomeric Aβ(1–40) is not able to bind to immobilized WO1 and WO2. Thus, Fig. 3 shows that although immobilized MAB1560 effectively binds N-terminally biotinylated Aβ(1–40), immobilized WO1 and WO2, as well as a control IgM mAb, are completely ineffective at binding this peptide.

Surprisingly, WO1 and WO2 are capable of binding not only to Aβ fibrils, but also to amyloid fibrils generated from other proteins. We generated amyloid fibrils from a number of amyloid precursor proteins (*Materials and Methods*). In each case the protein aggregates exhibit fibril or fibril-like structures in electron microscopy and a typical ThT fluorescence response (data not shown). Fig. 4*A* shows the binding of WO1 to equal weights of each of these amyloid fibrils immobilized onto microtiter plate wells. The figure shows that amyloid fibrils or amyloid-like aggregates composed of β2m, IAPP, TTR, polyGln (Q_{42}), the Ig V_L domain JTO5 (35), and lysozyme [in addition to Aβ(1–40)] are all capable of binding significant amounts of WO1. On a weight basis, WO1 binds different fibrils to different extents, exhibiting maximal binding to Aβ(1–40) fibrils. The binding differences observed may be due in part to differences in binding constants (see below) and/or to different numbers of epitopes per unit weight of these amyloid fibrils. WO2 exhibited similar pan-amyloid binding (not shown).

In contrast to its broad ability to bind to amyloid fibrils, WO1 exhibits little or no binding to the precursor proteins for each of these fibrils immobilized onto microtiter plate wells (Fig. 4*A*). This result suggests that the conformation recognized by WO1 is absent in the native precursor proteins. Fig. 4*A* also shows that WO1 binds to Aβ fibrils despite the presence of a high weight excess of soluble Aβ.

To probe the specificity of WO1 and WO2 for amyloid fibrils, we tested WO1 binding to different kinds of protein aggregates. Fig. 4*B* shows that WO1 does not bind appreciably to the fibrous proteins collagen and elastin, or to gelatin, the denatured form of collagen. Many globular proteins aggregate in response to disruption of their native states by chemical or physical stress, and these aggregates are often dominated by β-sheet structure (36). Fig. 4*B* shows, however, that WO1 does not bind to aggregates of ovalbumin or human serum albumin induced by denaturation via reduction and alkylation. Similar results were obtained for WO2 (not shown). These results show that the epitope(s) recognized by WO1 and WO2 is not a structural feature shared with amorphous protein aggregates, such as interchain β-sheet or diffuse patches of surface-exposed hydrophobicity. Thus, results to date suggest that the epitope recognized by these Abs is specific to the amyloid state of proteins.

To quantitatively assess the crossreactivity of WO1 and WO2 against other amyloid fibrils, we determined full binding curves for these Abs as well as for a control κ light chain IgM, against amyloid fibrils composed of Aβ(1–40), JTO5, and IAPP. Fig. 5*A* shows that WO1 and WO2 exhibit saturable binding curves to immobilized Aβ(1–40) fibrils (the original immunogen) with EC_{50}s of 2.8 nM and 1.3 nM, respectively. In contrast, the binding of a κ light chain control IgM to Aβ(1–40) fibrils exhibited a much weaker binding constant (121 nM) and a lower binding amplitude. The binding ability of this control IgM to Aβ fibrils

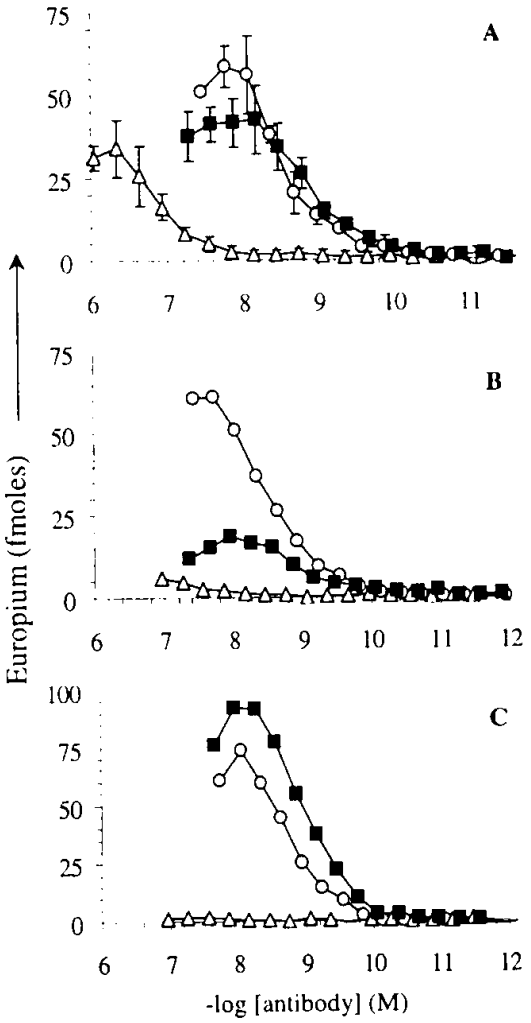

Fig. 5. Binding curves for purified WO1, WO2, and a control κ chain IgM against various amyloid fibrils immobilized onto microplate wells. WO1 (○); WO2 (■); and IgM control (△). (*A*) Aβ(1–40) fibrils. (*B*) Ig light chain variable domain JTO5 fibrils. (*C*) IAPP fibrils. Data in *B* and *C* are from single replicate determinations.

is probably related to the general ability of globular proteins to bind nonspecifically to Aβ fibrils (B.O.N. and R.W., unpublished observations). Interestingly, a λ chain control IgM is much less effective at binding to Aβ fibrils compared to the control κ chain IgM (data not shown).

Fig. 5*B* shows that WO1 and WO2 bind to JTO5 fibrils with EC_{50} values of 2.8 nM and 1.2 nM, respectively, values similar to those for binding to Aβ(1–40) fibrils. The amplitudes for WO1 and WO2 binding to JTO5 fibrils, although differing from each other by about a factor of three, are in the same range as the binding of these Abs to Aβ fibrils. The control κ light chain IgM exhibits essentially no binding to JTO5 fibrils. Fig. 5*C* shows that WO1 and WO2 also bind with similar amplitudes and binding constants (1.7 nM and 1.0 nM, respectively) to IAPP fibrils, whereas the control IgM binds negligibly. These data dramatically illustrate that WO1 and WO2 recognize what appears to be a universal amyloid epitope and that the ability to bind to amyloid fibrils is not shared by a control IgM. The Abs bind with similar strengths to amyloids other than the Aβ(1–40) amyloid immunogen. Although the IAPP peptide exhibits some amino acid sequence homology to Aβ(1–40) (P. Westermark, personal communication), JTO5 offers no significant homology. [This was confirmed by using the SIM (37), LALIGN (38), and DOTLET (39) sequence alignment programs (www.expasy.ch/tools) (data not shown)]. The nature of the common amyloid epitope, therefore, does not seem to depend on extensive amino acid side chain information. This point is made perhaps most dramatically by the fact that WO1 binds well to polyGln aggregates (Fig. 4*A*), which exhibit a number of amyloid-like features in their morphologies and biophysical properties (S. Chen, V. Berthelier, J. B. Hamilton, B.O.N., and R.W., unpublished data).

In their abilities to bind to multiple molecular species, WO1 and WO2 bear some resemblance to polyreactive Abs such as the anti-DNA Abs produced in certain autoimmune conditions. Although little is known about the basis for such polyreactive binding recognition, at least some anti-DNA Abs are thought to recognize repeat structures within DNA such as heteroaromatic bases or phosphate groups (40, 41). As highly ordered polymers, amyloid fibrils presumably also exhibit certain kinds of regular structural repeats that might serve as the basis for multidentate-binding recognition. The extent to which WO1 and WO2 binding to amyloid might depend on such multidentate binding is yet to be determined.

One possible contributor to a common amyloid structural epitope might be a unique array of H-bond donor and acceptor groups from the polypeptide backbone at the edge strands of the β-sheets on the ends of amyloid fibrils. If so this configuration must be different from that of the edge strands of β-sheets in the native states of β-sandwich-based proteins such as the F_V domain and TTR because these globular, native proteins do not bind WO1 and WO2 appreciably (Fig. 4). Another possibility is main chain elements involved in some unusual turn or chain reversal within the amyloid motif. Detailed structures of the epitopes of anti-protein Abs are normally characterized either by protein crystallography (42) or by mutational analysis of antigen fragments (43) or intact protein (44). Because amyloid has yet to be crystallized, and because WO1 and WO2 bind to many amyloids regardless of amino acid sequence, it is clear that the further structural analysis of the WO1/WO2 epitope(s) will be challenging.

Aβ(1–40) fibrils are not unique in their ability to stimulate production of generic anti-amyloid Abs in mice. For example, a mAb raised against an amyloidogenic Ig light chain fragment has been reported to recognize tissue amyloid deposits composed of the light chain variable domain, Aβ, and other amyloidogenic proteins (13). The reciprocity of these results with those described here further supports the existence of an epitope that is a universal signature of the amyloid fibril.

Abs such as WO1 and WO2 are important for a number of reasons. They may prove invaluable in improving our knowledge of the three-dimensional structures of amyloid fibrils. Such Abs will also allow us to monitor more closely the generation of the amyloid epitope during assembly *in vitro* and *in vivo*. Fibril-specific Abs may be useful as passive vaccines in anti-amyloid therapy (13, 15, 16, †). Furthermore, recognition that an amyloid fibril from one precursor protein can stimulate a pan-amyloid response in an animal suggests that amyloid fibrils composed of one constituent protein might serve as vaccines against other types of amyloid. Finally, Abs such as WO1 and WO2 may prove to be useful diagnostic reagents not only for Alzheimer's disease, but also for other protein aggregation diseases. The defining clinical test for the presence of amyloid in tissue continues to be birefringence after Congo red staining (12). However, as a technique that is inherently limited in resolution to the wavelength of visible light, Congo red birefringence is not capable of detecting deposits of small and/or disorganized fibrils if they do not exhibit macroscopic order. Abs such as WO1 and WO2

may thus prove to be more reliable probes for the presence of the amyloid-folding motif in tissue samples.

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TREATMENT OF AMYLOIDOSIS USING AN ANTI-FIBRIL MONOCLONAL ANTIBODY: PRE-CLINICAL EFFICACY IN A MURINE MODEL OF AA-AMYLOIDOSIS

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Introduction

The amyloidoses are a diverse spectrum of diseases which share a common pathologic manifestation; namely, the formation of fibrils composed of normally innocuous, soluble proteins or peptides that deposit in various tissues throughout the body eading to organ failure and eventually death. Therapeutic efforts to prevent or cure these diseases have focused primarily on inhibiting fibrillogenesis or eliminating the precursor protein. More recently, immune-based strategies have been formulated and have been shown experimentally to have therapeutic potential[1,2]. We have reported the development of anti-light chain monoclonal antibodies (mAbs) that have the capacity to recognize an amyloid-associated epitope present on AL and other types of amlyoid[3]. When administered *in vivo* such reagents were found to accelerate the removal of AL deposits in an experimental animal model[3]. We now report one such mAb designated 11-1F4 hastens the resolution of amyloid in mice with AA-amyloidosis.

Materials and Methods

In vivo studies were performed using a murine model of inducible AA-amyloidosis. C57/Bl mice were primed by administering two 1 mL *s.c.* injections of 0.1% (w/v) $AgNO_3$ solution, 10 d. apart. Amyloidogenesis was induced by the *i.v.* injection of amyloid enhancing factor (AEF; 0.1 mg/mL protein) coincident with the first $AgNO_3$ injection (day 0). The following treatment regimen was used: administration of 11-1F4 mAb, began on day 16 and was repeated on days 19, 23 and 26. The mice were sacrificed on day 28 and the effects of 11-1F4 mAb-treatment were determined by quantitative evaluation of the splenic and hepatic amyloid burden, i.e., the Amyloid Burden Index (ABI). Tissues sections were collected post-mortem, fixed, paraffin-embedded, and stained using alkaline

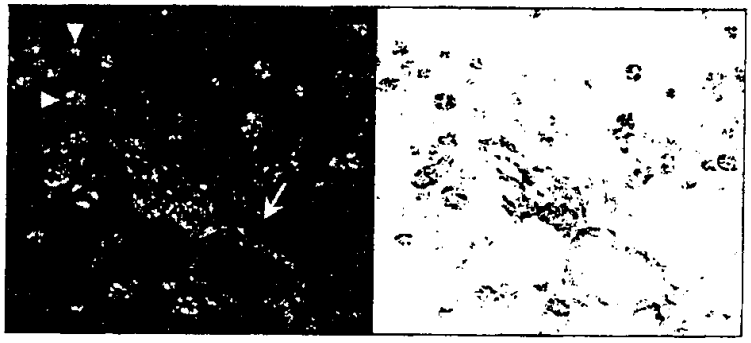

Figure 1. Identification of amyloid using image analysis. Left, CR-stained section of AA-laden liver. Right, identification of amyloid (white) based upon CR birefringence

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Congo red. When viewed microscopically under cross-polarized illumination the amyloid deposits exhibited characteristic blue-green birefringence, which was then quantified using image analysis and spectral segmentation techniques yielding the ABI (Fig. 1).

Results

Image analysis revealed a marked decrease in the ABI of mice treated with mAb 11-1F4 after induction of AA-amyloidosis (table 1). Quantification of amyloid deposits was achieved by image analysis of CR-stained tissue sections. The identification of AA-amyloid in the liver of an AEF/$AgNO_3$-treated mouse is shown in Fig. 1. The section contained both perivascular amyloid and deposits within the lumen of the sinusoids that exhibited the characteristic "Maltese-cross" pattern of CR-birefringence (Fig. 1). The amyloid deposits were accurately detected using the image segmentation software and the spectral profile, while the collagenous regions were excluded (Fig. 1). This technique provided a method to quantitatively evaluate the effect of 11-1F4 mAb treatment on amyloid burden (Table 1). The population data presented in box-plot format (below, Fig. 2) demonstrated significant decreases in the range, median and mean of the ABI within the spleen, as a result of treatment with the 11-1F4 mAb. The quantitative effects of antibody treatment on amyloid burden were considerably more evident within the spleen than the liver, which appeared to exhibit similar statistical parameters irrespective of treatment, with no significant difference in the mean or median of the two populations (Table 1).

	Untreated Mice (*n*=7)		**11-1F4 antibody-treated Mice (*n*=7)**	
	Liver	*Spleen*	*Liver*	*Spleen*
Mean ABI (% ± S.E.)	0.211 ± 0.141	0.903 ± 0.397	0.052 ± 0.025	0.190 ± 0.071
% Reduction in Mean ABI	-	-	75.4%	79.0%*
ABI Range	1.044	2.918	0.179	0.513
ABI Median	0.057	0.420	0.029	0.132

Table 1. Comparison of the hepatic and splenic amyloid burden indices (ABI) of 11-1F4 antibody-treated and control mice. ABI percentage values represent the area occupied by amyloid deposits within the tissue relative to the total area surveyed (approximately 22 mm^2 total area), * $P < 0.075$.

Discussion

Treatment of mice with AA amyloidosis using the 11-1F4 mAb resulted in a marked decrease in the amyloid content of both the liver and spleen. Immunotherapeutic approaches to the treatment of amyloidosis have had considerable success in murine models of both AL amyloidomas[3] and Alzheimer's disease[1,2]. However, there are several considerations. Antibodies designed to target amyloid fibrils, may also interact with the soluble form of the precursor protein and potentially the protein producing cells, an assault that may ultimately lead to severe complications such as auto-immunity and immune-complex disease. These considerations may not be problematic for localized

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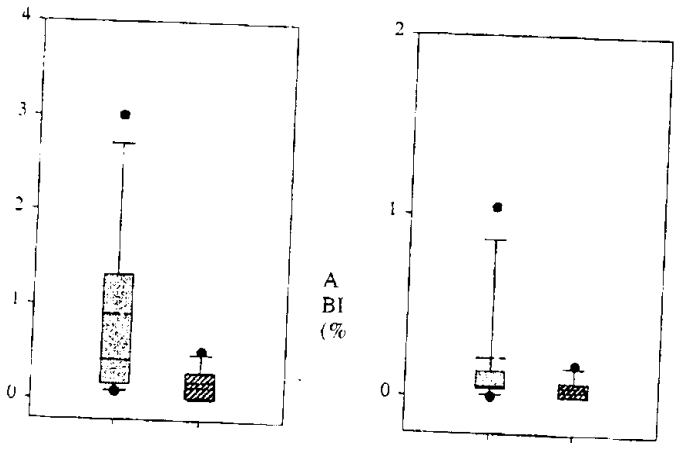

Figure 2. Comparison of hepatic and splenic amyloid burden indices (ABI) for 11-1F4 treated and controls. The dashed and solid lines are the mean and median, respectively. The boxes represent the 25th and 75th percentile and the error bars the 10th and 90th percentiles.

cerebral amyloids. However, for non-cerebral, systemic and localized forms of amyloidosis derived from otherwise soluble proteins, present at high concentrations in the circulation, these considerations are paramount. It is anticipated that the use of amyloid-specific antibodies, such as 11-1F4, will effectively facilitate the resolution of amyloid deposits and circumvent issues of autoimmunity, and provide a viable therapeutic approach for the treatment of this family of devastating diseases.

References

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